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14. ABSTRACT

BACKGROUND: Combat wounds have unique features including significant devitalization due to high-energy blast, deep contamination, complex/composite tissue loss, and multiple coexisting injuries. In this regard, Composite Tissue Allotransplantation (CTA) is an innovative reconstructive modality that can provide functional restoration after complex musculoskeletal trauma. CTA is now a clinical reality with numerous upper extremity and face transplants performed worldwide thus providing new hope for service members suffering from catastrophic combat trauma and devastating tissue defects. Broader clinical application of CTA, however, continues to be hampered by requirement for long-term multi-drug immunosuppression to prevent graft rejection. This study for the first time proposes a novel cell-based therapy utilizing Mesenchymal Stem Cells (MSC) that augments nerve regeneration while minimizing the need for immunosuppression.

METHODS: Sciatic nerve transections and repairs were performed on Lewis rats in control and experimental groups with local and systemic (intravenous) MSC injection *(n=8 per group).* Syngeneic (Lewis-Lewis) and Allogeneic (Lewis-Brown Norway) hind limb transplants were performed to analyze neuroregenerative effects of MSC with and without allo-immune response *(n=4 per group).*

RESULTS: Compound Muscle Action Potential (CMAP) amplitudes significantly improved for systemic MSC injection groups relative to controls after sciatic nerve transection and repair. However, no significant differences were observed between groups in functional gait analysis. In addition, histomorphometry and gastrocnemius weight data did not indicate any significant differences among groups. However, the number and density of axons observed between 6 and 16 weeks increased by approximately 35% and 30%, respectively.

Syngeneic hind limb transplants that received local or systemic MSC injections showed significant enhancement of functional recovery following MSC therapy. Catwalk gait analysis indicated an improvement in paw positioning and intensity relative to control in the local MSC injection group at 16 weeks. Alternatively, the systemic MSC injection group was noted to have a significant increase in average maximum CMAP relative to controls at 16 weeks. Although not statistically significant, there was a clear objective trend toward improved function in groups that received MSC injections.

Allogeneic transplants were performed with local and systemic MSC injection along with a short course of immunosuppression for 30 days. After cessation of immunosuppression, both experimental groups and control animals rejected the allograft within 2 weeks due to highly immunogenic skin component. No significant differences were noted on histology or gastrocnemius weight at this early time point. Additionally, due to the early rejection of the grafts, there was no significant functional recovery noted on electromyography or Catwalk gait analysis. However, *in vitro* mixed lymphocyte reaction showed potent immunoregulatory properties of MSCs.

CONCLUSION: Mesenchymal Stem Cells enhance functional recovery following nerve injuries and limb transplantation. Mesenchymal stem cells also possess potent immunomodulatory properties with minimal immunogenicity *in vitro*. However, due to highly immunogenic skin component of CTA in a stringent fully mismatched strain combination, the immunomodulatory properties could not be demonstrated *in vivo*. Further studies are warranted to explore mechanistic relationships of MSC therapy in nerve regeneration and to optimize dosage and frequency of MSC therapy for targeted immunomodulation in CTA.

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MSC Therapy for Nerve Regeneration and Immunomodulation after Composite Tissue Allotransplantation

INTRODUCTION

Combat wounds have unique features including significant devitalization due to high-energy blast, deep contamination, complex/composite tissue loss, and multiple coexisting injuries. Current treatment options like nerve grafts, nerve transfers, and synthetic nerve conduits are limited by the wide zone of injury, large nerve gaps and lack of available autologous nerve secondary to massive tissue trauma. Devastating musculoskeletal and extremity trauma constitute a major portion of combat injuries where functional outcomes after multiple conventional reconstructions and delayed nerve repair are suboptimal.

In this regard, Composite Tissue Allotransplantation (CTA) is an innovative reconstructive modality that can provide functional restoration after complex musculoskeletal trauma such as upper extremity amputation or disfiguring facial injuries[1]. CTA is now a clinical reality with numerous upper extremity and face transplants performed worldwide thus providing new hope for service members suffering from catastrophic combat trauma and devastating tissue defects. Broader clinical application of CTA, however, continues to be hampered by requirement for long-term multi-drug immunosuppression to prevent graft rejection. Medication toxicity could result in metabolic and infectious complications or malignancy. Furthermore, clinical success in CTA is dictated not only by graft acceptance, but also by functional outcome, which depends on degree and quality of nerve regeneration. The implementation of cellular therapies that integrate the concepts of immune regulation with those of nerve regeneration can optimize the functional outcomes of these reconstructive modalities and minimize the need of immunosuppression and thereby significantly favor the risk-benefit-ratio.

Bone marrow derived mesenchymal stem cells (BM-MSCs) are pluripotent cells, capable of differentiation along multiple mesenchymal lineages into osteocytes, chondrocytes, myocytes, and adipocytes[2]. Recent advances have shown that MSC can also trans-differentiate into Schwann cells (SC)[3]. MSC-enhanced nerve regeneration has been demonstrated both *in vitro* and *in vivo*. Other than bone marrow, MSCs are also present in adipose tissue, skin, heart and placenta and can be isolated and expanded *ex vivo* thereby emerging as a promising tool for cell based therapeutic strategy[4]. Recently, BM-derived MSCs have been identified to have potent immunosuppressive properties. Most importantly, MSCs are considered to be immunoprivileged by their low immunophenotype[4-5]. MSC offer some potential advantages over conventional immunosuppressive agents by specifically targeting immunoinhibitory effects that could prevent rejection, and minimize the systemic complications of nonspecific immunosuppressant in the setting of CTA[6-7]. In addition to the immunomodulatory effects of MSC, they have demonstrated the ability to prevent and treat GVHD[8], one of the most serious complications following transplantation.

This study for the first time proposes a novel cell-based therapy utilizing MSCs that augments nerve regeneration while minimizing the need for immunosuppression.

BODY

TASK 1: Demonstrate neuroregenerative effect of systemic MSC in a sciatic nerve transection model.

MILESTONE 1A: Establish MSC Culture Protocol.

BM-MSC HARVEST AND FUNCTIONAL CHARACTERIZATION:
Bone marrow derived MSCs were harvested from femur and tibia of anesthetized adult Brown Norway rats (4-6 weeks old). BM-MSCs were isolated based on their inherent plastic adherence when grown in culture media. After 2-3 passages, MSCs were further purified via FACS sorting.

Immunophenotypic characterization was performed by flow cytometric analysis. MSCs consistently and homogenously expressed CD29 and CD90 and were negative for CD11, CD45, RT1A and RT1B (Figure 1).

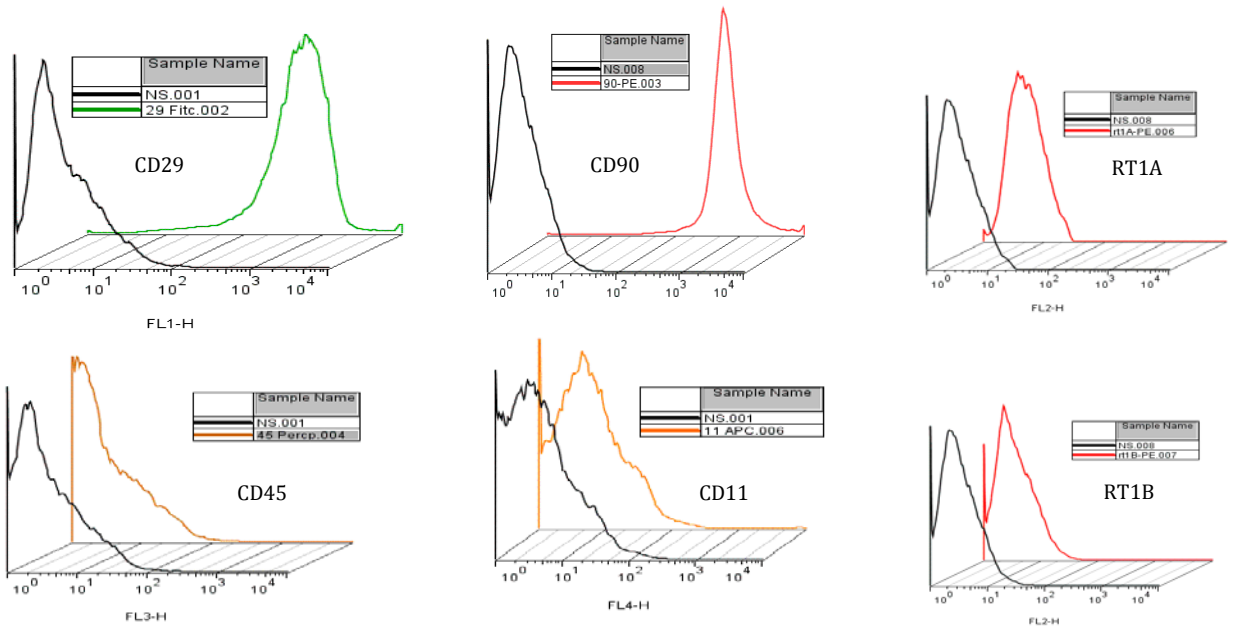


Figure 1: BM-MSCs consistently and homogenously expressed cell surface markers CD29 and CD90 and were negative for CD45, CD11, RT1A and RT1B as determined by flow cytometry analysis using fluorescent labeled monoclonal antibodies.

The culture-grown BM-MSCs were tested for their ability to differentiate into adipocytes, osteoblasts, and chondrocytes: Osteoblasts were identified by von Kossa staining, adipocytes by oil-red O staining, and chondrocytes by Alcian blue staining (Figure 2).

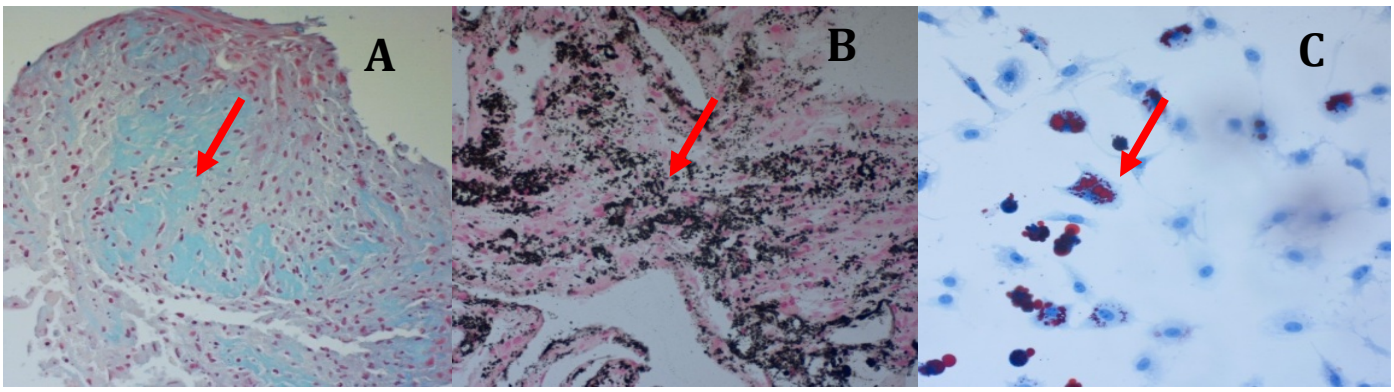


Figure 2: Differentiation Potential of BM-MSCs: (A) Chondrogenesis: arrow indicated blue-stained deposition with Alcian-Blue, suggesting the formation of collagen, (B) Osteogenesis: With Von Kossa Staining, the cytoplasmic calcium deposition was shown as black deposition (Arrow) and (C) Adipogenesis: Oil-O Red staining showed the intracellular lipid-filled vesicles

MILESTONE 1B: Neuroregenerative Effects of BM-MSCs in Sciatic Nerve Transection and Repair Model.

SCIATIC NERVE TRANSECTION AND REPAIR:
As a control group, eight animals received sciatic nerve transection with suture repair. No cells were injected in this group. In the "Local" and "Systemic" groups, eight animals each received sciatic nerve transection with either local administration of MSCs into the distal stump or a systemic (IV) injection of MSCs, respectively. The three groups of eight were then further subdivided into a 6 week and 16 week measurement group (Table 1)

Group	Experiment	Treatment	Endpoint
I (Control)	Sciatic Nerve Transection and Repair	No treatment	6 weeks *(n=4)*
			16 weeks *(n=4)*
II	Sciatic Nerve Transection and Repair	Local MSC	6 weeks *(n=4)*
			16 weeks *(n=4)*
III	Sciatic Nerve Transection and Repair	Systemic MSC	6 weeks *(n=4)*
			16 weeks *(n=4)*

.
All Sciatic Nerve Transection and Repair procedures were performed in a similar fashion. A gluteal skin incision was made from the area of sciatic notch to just above the knee joint. The gluteal muscles were separated in order to expose the Sciatic nerve from the sciatic notch to the point of bifurcation. Then, the nerve was transected and approximated with interrupted epineurial 10-0 nylon suture (Figure 3). All animals in the local injection groups received 50,000 MSCs injected into the distal nerve stump using a 33-gauge needle attached to a custom made 50-µl syringe. All animals in the systemic groups received

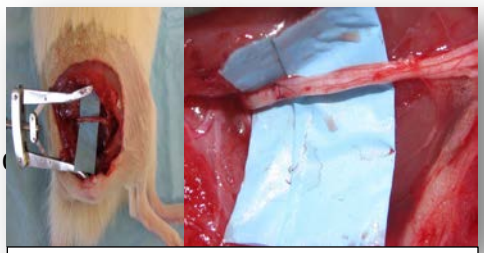


Figure 3: Sciatic nerve transected and repaired primarily using 10-0 epineureal suture

10^6 MSCs injected into tail vein at the end of procedure.

COMPUTER-ASSISTED GAIT ANALYSIS USING CATWALK:

Catwalk XT System (Noldus Technology). The CatWalk XT provides advanced gait analysis in rodents. The system consists of an enclosed walkway, a high-speed color camera, and recording and analysis software to assess the locomotor performance of rodent models (Figure 4). While animals traverse the walkway from one side to the other side in a non-enforced manner, their footprints are captured with a high-speed video camera. The video camera sends the capture to a computer that runs the CatWalk XT software. Utilizing Illuminated Footprint technology, the paw print area, contact intensity, swing speed and swing distance are captured. From this data, numerous parameters are calculated for qualitative and quantitative analysis of individual footfalls and gait. In order to provide consistent and reproducible functional outcome data, we introduced pre-operative training of the rats on the CatWalk system. As a result, their walk through the CatWalk walkway became unforced, continuous and consistent, leading to greater accuracy in the classification of their walking behavior. In addition, the calibration settings of the CatWalk were optimized to increase the detection accuracy of the machine. Pilot experiments were conducted involving various models of rat peripheral nerve injuries (sham, nerve gap, crush injury, transection and repair) to confirm reproducibility of data. After optimization, we recorded bi-weekly functional outcome data on the MSC injection experimental groups. Functional gait analysis was performed to compare between experimental groups. A selection of the most relevant components of this data is shown in Figure 5. Of the 22 parameters examined, no significant differences were noted for any of the major primary outcomes by difference of means. Specifically, there was no improvement in the strength or pattern of experimental paw placement for experimental groups relative to control. Terminal dual stance, however, a measure of final postural positioning, was significant for the local MSC injection group relative to control (p=0.035).

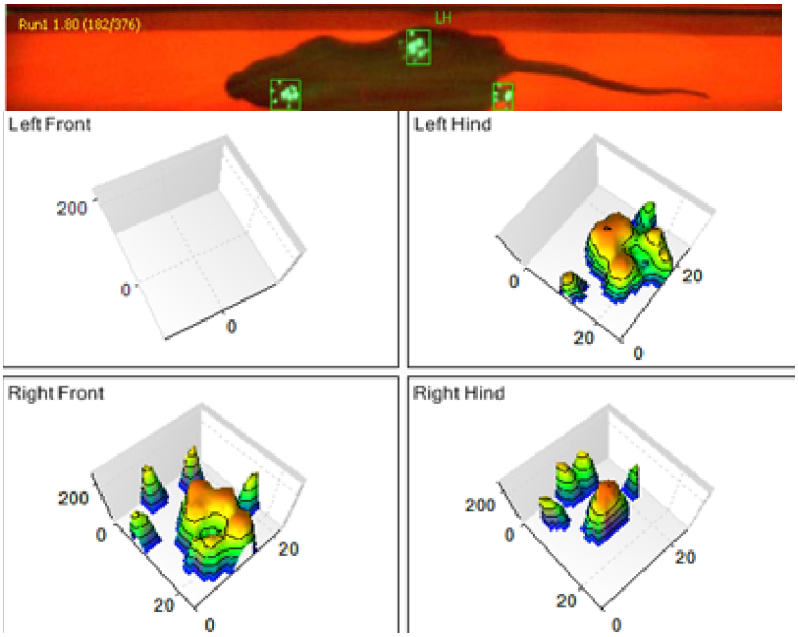


Figure 4: CatWalk XT (Noldus Technology): Locomotor performance is assessed utilizing a high-speed video camera for footprint capture and computer-assisted qualitative and quantitative functional analysis.

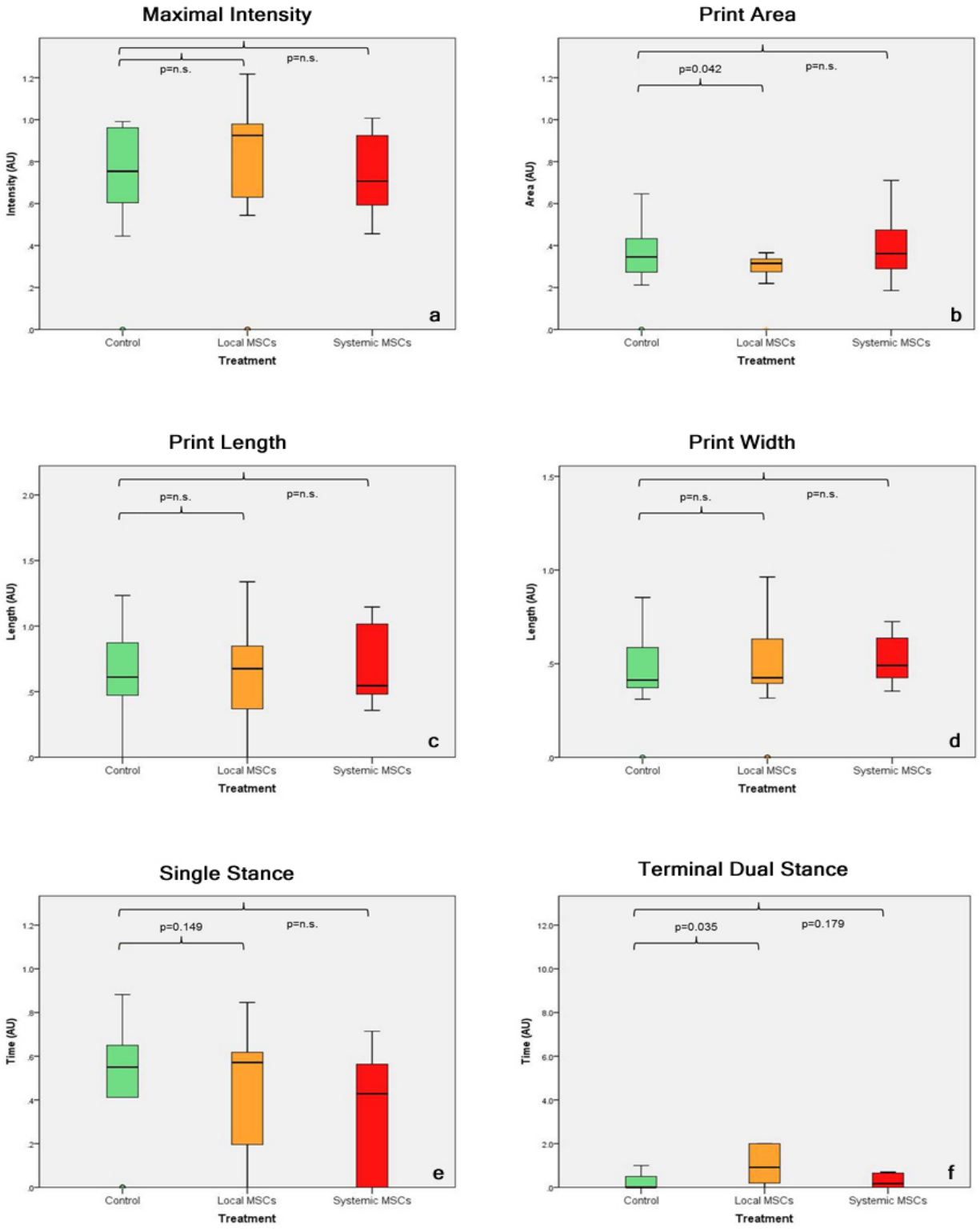


Figure 5: Functional Outcome Analysis following Sciatic Nerve Repair (at 16 weeks): Analysis of gait parameters using the CatWalk system revealed no significant difference in Print Area, Maximal Contact Intensity, Print Length, Print Width and Single Stance among Control, Local MSC and Systemic MSC therapy Groups. Terminal Dual Stance, however, showed significant improvement in

local MSC therapy Group (p=0.035). The first four parameters evaluate paw placement and position. The final two parameters indicate animal posture.

ELECTROPHYSIOLOGY:

Using an ADInstrument's electrophysiology system (Colorado Springs, CO), we optimized highly sensitive compound muscle action potential (CMAP) recordings (Figure 6). The CMAPs were measured in the intrinsic foot muscles on the plantar surface using sub-dermal needle electrodes. Serial CMAP measurements were performed on a bi-weekly basis. The non-operated, contralateral side serves as the intrinsic control for the maximum attainable CMAP in a given rat.

Recorded maximal amplitude and latency data normalized to the contralateral, non-operated, limb is shown in Figure 7 and 8. Data is shown for changes in normalized EMG data every four weeks until 16 weeks after nerve transection and repair. Amplitude was measured as the maximal deflection from baseline, and latency was measured as the time from stimulation to this maximal deflection point.

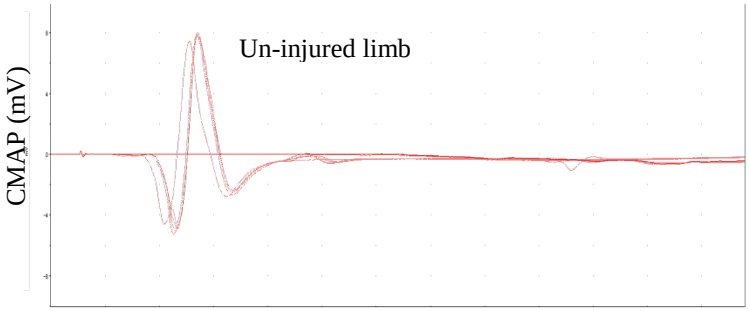


Figure 6: CMAP (mV) recordings from un-injured limb. Stimulating electrodes were placed near the sciatic notch and recordings electrodes were placed in intrinsic muscles of footpad.

A clear trend towards improvement in amplitude was observed in all groups (see Figure 7). Moreover, difference of means analysis indicated that systemic administration of MSCs resulted in higher CMAP amplitudes at 12 and 16-week time points relative to controls (p = 0.004 and 0.1, respectively). Similarly, a decrease in CMAP latencies was observed over time but differences among groups were not statistically significant.

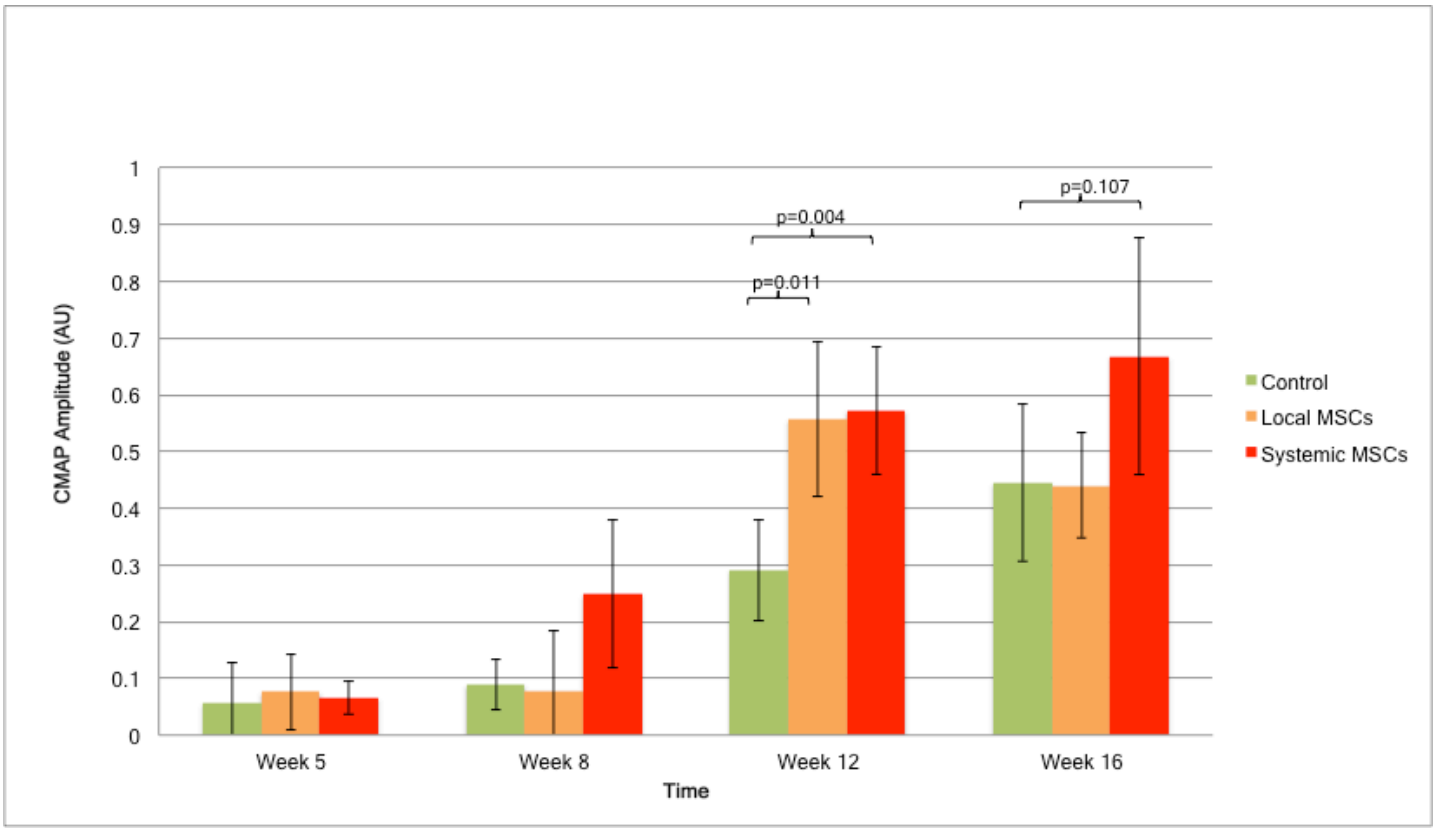


Figure 7: Sciatic Nerve Transection and Repair: Normalized Experimental CMAP Amplitudes Over time.

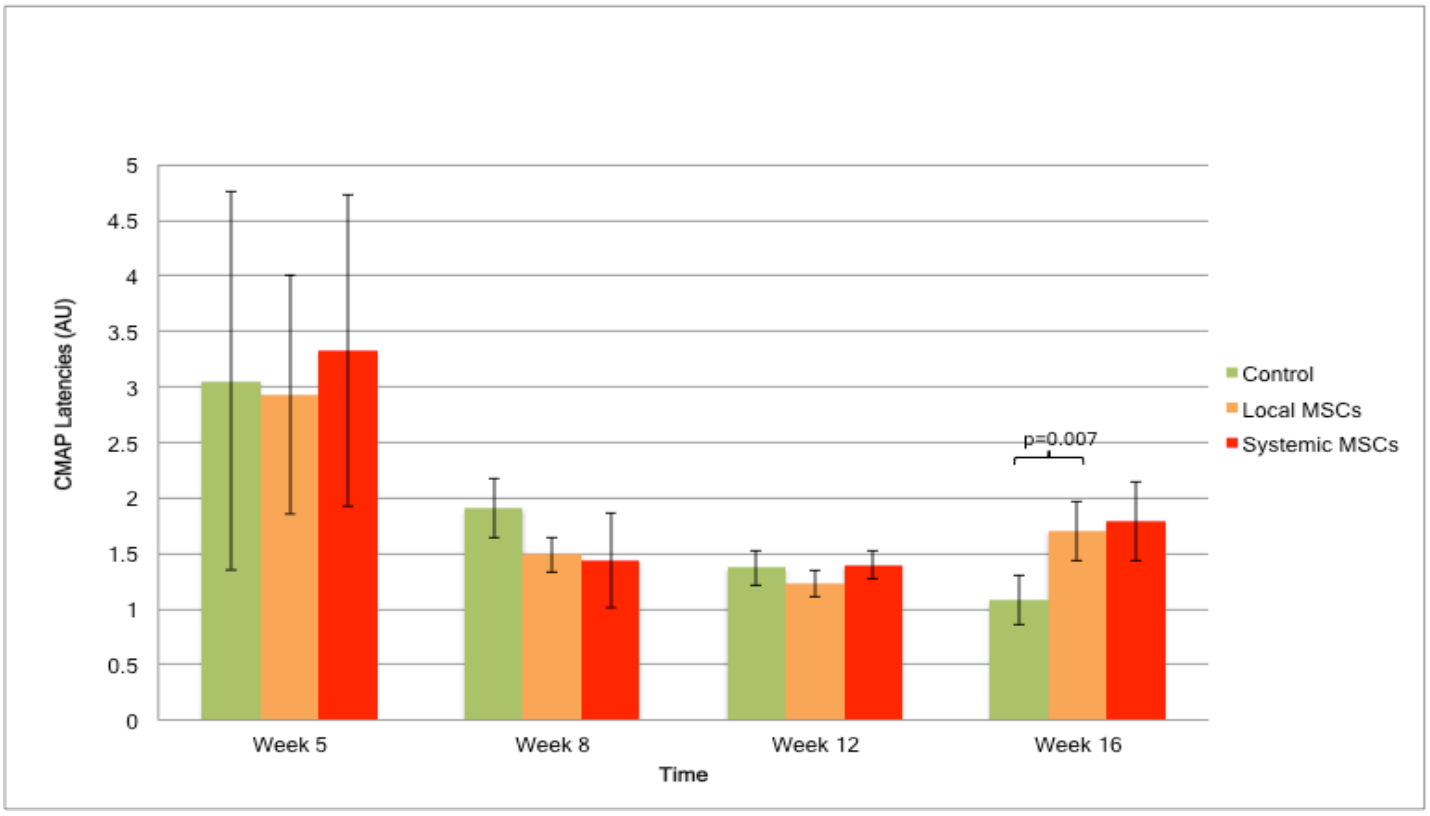


Figure 8: Sciatic Nerve Transection and Repair: Normalized Experimental CMAP Latencies Over Time.

NORMALIZED GASTROCNEMIUS WEIGHT ANALYSES:

Gastrocnemius weights — a proxy for improved nerve regeneration or reduce atrophy were measured for non-operated contralateral limbs and for the experimental limbs in all groups. Results are shown in Figure 9 with normalization of experimental limb gastrocnemius weight to contralateral non-operated limb. Systemic MSC injection group showed a trend toward an increase in muscle weight, but was not significantly different when compared using difference of means testing (p=0.071).

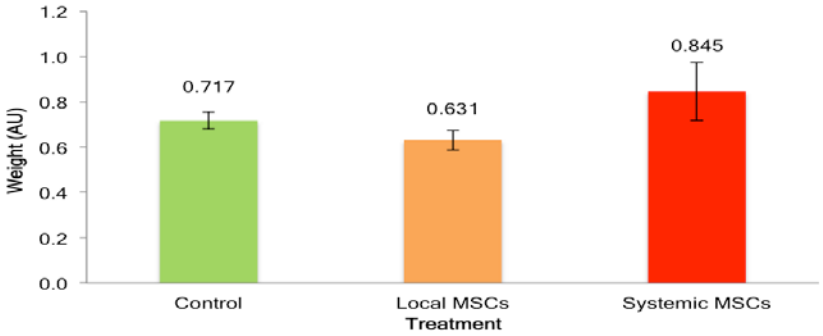


Figure 9: Sciatic Nerve Transection and Repair: Normalized Gastrocnemius Muscle Weights at 16 weeks

NERVE HISTOMORPHOMETRY:

We optimized histomorphometric analysis of nerve sections for objective measurement of axonal regeneration. Briefly, a nerve section 5-8 mm distal to the anastomosis site was fixed in glutaraldehyde, post-fixed with osmium tetroxide, and embedded in resin. Five-micrometer thick cross-sections were cut and stained with toluidine blue for examination using light microscopy. Figure 10 shows representative optimized stains from our experimental groups. At 100x

magnification, 5-7 randomly selected fields per nerve were evaluated for myelinated axon counts and fiber area. From these, total numbers of myelinated axons were measured per nerve fiber and nerve axon density (axons/mm^2) was calculated. An investigator blinded to experimental groups performed all measurements. In order to better elucidate the differences in early and late phases of nerve regeneration, sub-groups of experimental and control data were collected at 6 and 16 weeks. Histomorphometric analysis of 6 and 12 week data is depicted in Figure 11 and 12, respectively. As expected, the number and density of axons increased by an average of 35% and 30%, respectively, when comparing the two time points among groups. Relative to the local and systemic MSC injection groups, the control group qualitatively showed smaller numbers of total regenerating axonal sprouts and a lower overall density at early and late time points. However, a T-test for differences of means did not reveal any statistically significant differences relative to the control group. This is likely due to expected variability in the regenerating axons.

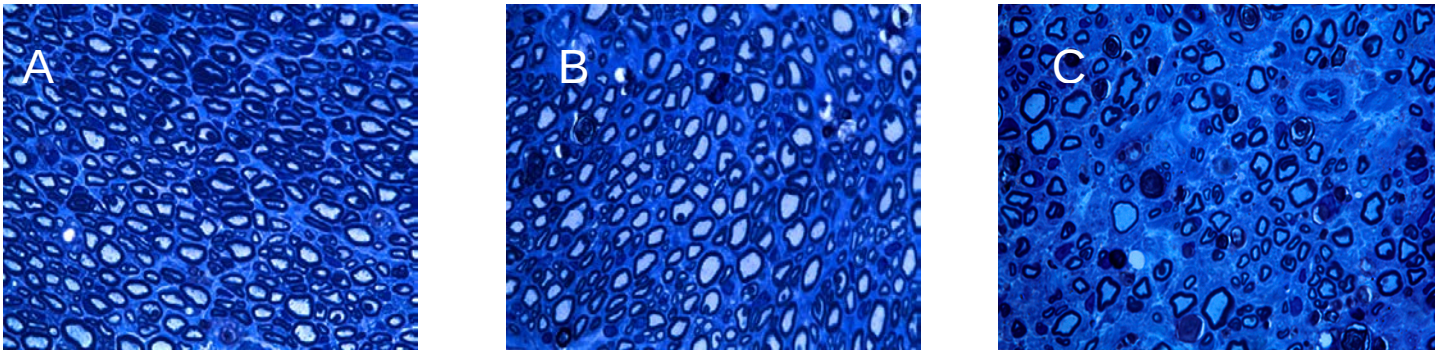


Figure 10: Light Microscopic Image (100X, stained with Toluidine Blue): Nerve Cross Section 5-8 mm distal to anastomosis site. Representative images from (A) Systemic MSC therapy, (B) Local MSC therapy and (c) No treatment Control

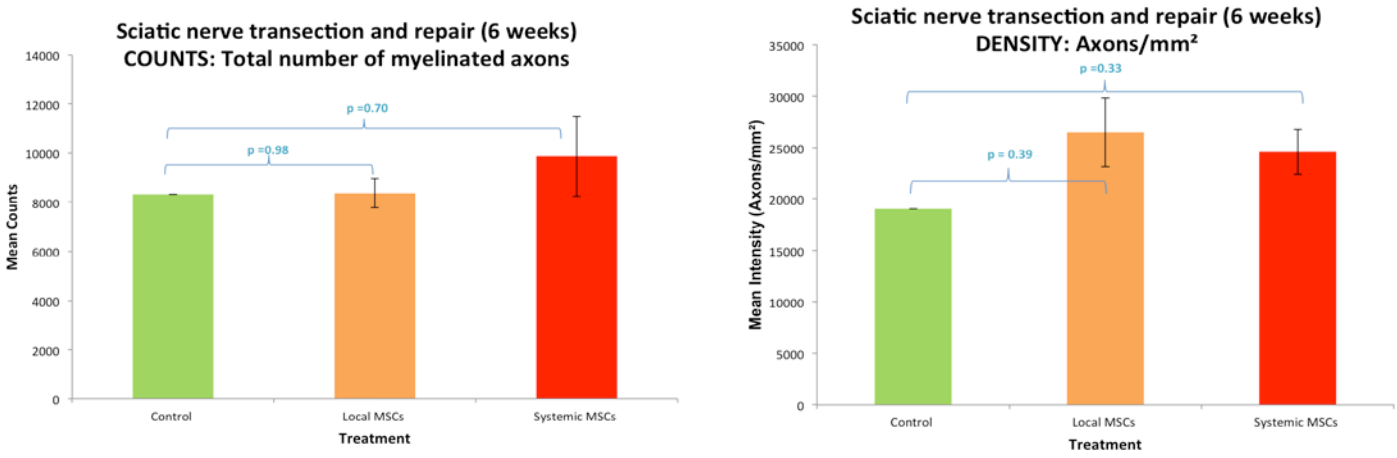


Figure 11: Sciatic Nerve Transection and Repair (6 weeks): A comparison total count of myelinated axons and axon density among control, local MSC and systemic MSC treatment groups. No statistically significant difference was found for experimental groups relative to controls.

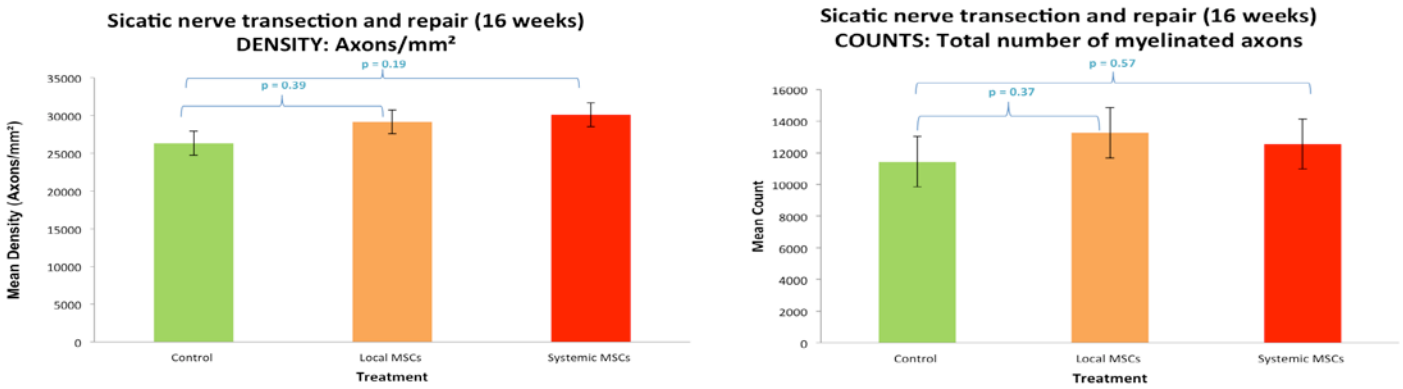


Figure 12: Sciatic Nerve Transection and Repair (16 weeks): A comparison total count of myelinated axons and axon density among control, local MSC and systemic MSC treatment groups. No statistically significant difference was found for experimental groups relative to controls.

Overall Summary:

Sciatic nerve transections and repairs were performed on control groups and experimental groups with local and systemic MSC injection. Functional recovery data indicated that CMAP amplitudes objectively improved in all groups over 16 weeks. At this final time point, CMAP amplitudes significantly improved for systemic MSC injection groups relative to controls. This improvement was not observed in Catwalk gait analysis. No major differences were recognized between groups in functional gait as all groups seemed to improve proportionally over time. In addition, histological differences were not observed. Histomorphometry and gastrocnemius weight data did not indicate any notable differences among groups. However, the number and density of axons observed between 6 and 16 weeks increased by approximately 35% and 30%, respectively. The discrepancy between functional and histological differences is likely due to large inter-animal variability and small numbers that are not related to actual nerve function. Overall, given the significant changes seen on EMG in this controlled environment, we are pursuing more complex analysis of histological data to uncover more nuanced features of nerve data that may be related to function.

Task 2: Determine neurogenerative effects of MSC therapy in Syngeneic Hind Limb Transplant

Hind limb Transplant:

We optimized our orthotopic hind limb transplantation model and then performed transplants from a Lewis-to-Lewis rat (syngeneic transplant) or Brown Norway to Lewis rat (allogeneic transplant, Figure 13). In brief, the femoral nerve, artery and vein were isolated and divided ensuring adequate length for subsequent anastomoses. The remaining thigh muscle groups as well as the sciatic nerve were transected to expose the mid-portion of the femur. A transverse osteotomy was performed through the femur to complete allograft harvest. The recipient animal was prepared in a similar fashion. Transplantation of the allograft was performed with osteosynthesis of the femur. The femoral vein and then femoral artery were anastomosed. The sciatic as well as the femoral nerve were approximated with interrupted epineurial 10-0 nylon sutures. The ventral and dorsal muscle groups were then repaired with 4-0 Vicryl prior to skin closure with 4-0 Ethilon. Cell delivery method remained the same as described for Sciatic Nerve Injury Model.

Figure 13: Allogenic Hind Limb Transplant: Orthotopic hind limb transplant from Brown Norway to Lewis Rat

Animals undergoing syngeneic hind limb transplants were divided into four groups: a no treatment control group; a short-term immunosuppression control group (0.5mg/Kg daily tacrolimus for 30 days); a local MSC experimental group ($50x10^4$ cells injected into distal stump with tacrolimus for 30 days); and a systemic MSC experimental group (single dose of 10^6 million cells IV intra-operatively with tacrolimus for 30 days). Tacrolimus was administered in one control group and both experimental groups to provide a consistent comparison between the syngeneic transplants performed in this task and the allogeneic transplants performed in the following task.

Functional and histological methods to assess efficacy of our treatment regimens remained the same as previously described.

COMPUTER-ASSISTED GAIT ANALYSIS USING CATWALK:

Computer-assisted gait based analysis showed marked differences among various groups. For the purposes of Catwalk analysis, control groups with and without tacrolimus were conflated under a

common control group. A selection of relevant parameters is shown in Figure 14.

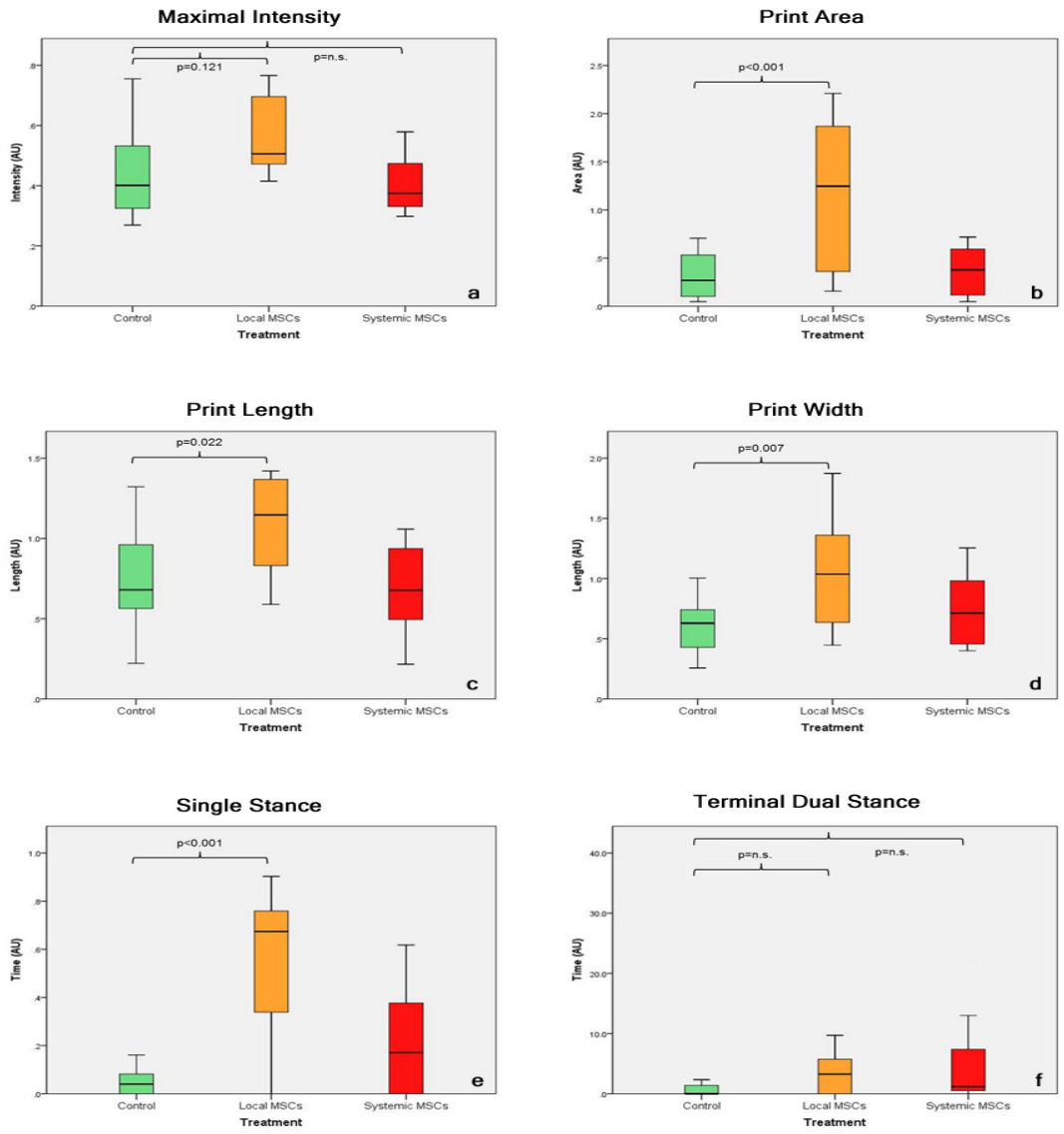


Figure 14: Functional Outcome Analysis following Syngeneic Hind Limb Transplant (at 16 weeks): Analysis of gait parameters using the CatWalk system revealed significant differences in Print Area, Maximal Contact Intensity, Print Length, Print Width and Single Stance for MSC therapy groups when compared no cellular treatment control.

Analysis of 22 individual functional parameters showed significant differences among 5 relevant gait characteristics using difference of means testing. Specifically, paw print length, width, area, and intensity were all significantly increased for the local MSC injection experimental group relative to controls. No significant differences were noted between the systemic injection group and controls.

ELECTROPHYSIOLOGY:

A clear trend towards improvement in amplitude over time was observed in all groups. Furthermore, systemic administration of MSCs resulted in higher CMAP amplitudes at 16-week time points ($p = 0.089$) when compared to controls (T-test) (Figure 15). Similarly, a decrease in CMAP latencies was observed over time, but differences among groups were not statistically significant for both parameters (Figure 16).

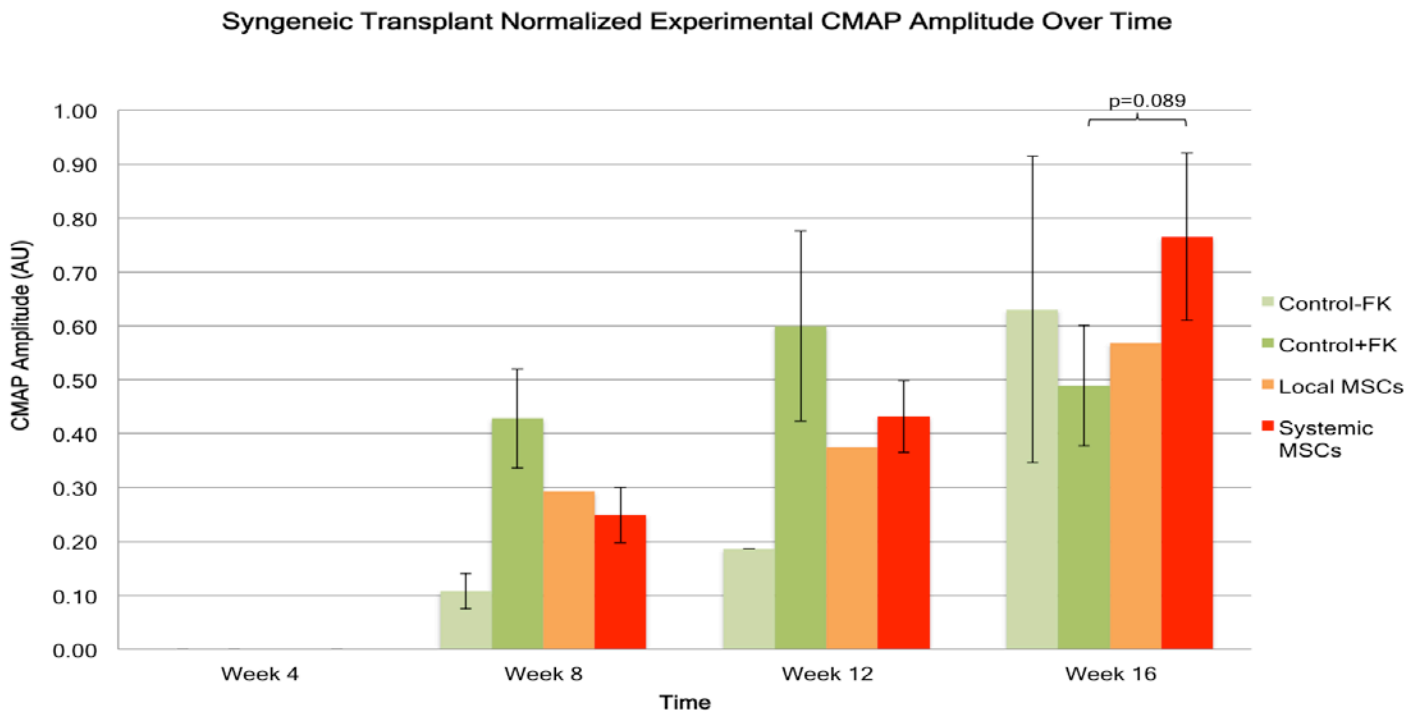


Figure 15: Syngeneic Hind Limb Transplant: Normalized Experimental CMAP Amplitudes Over time.

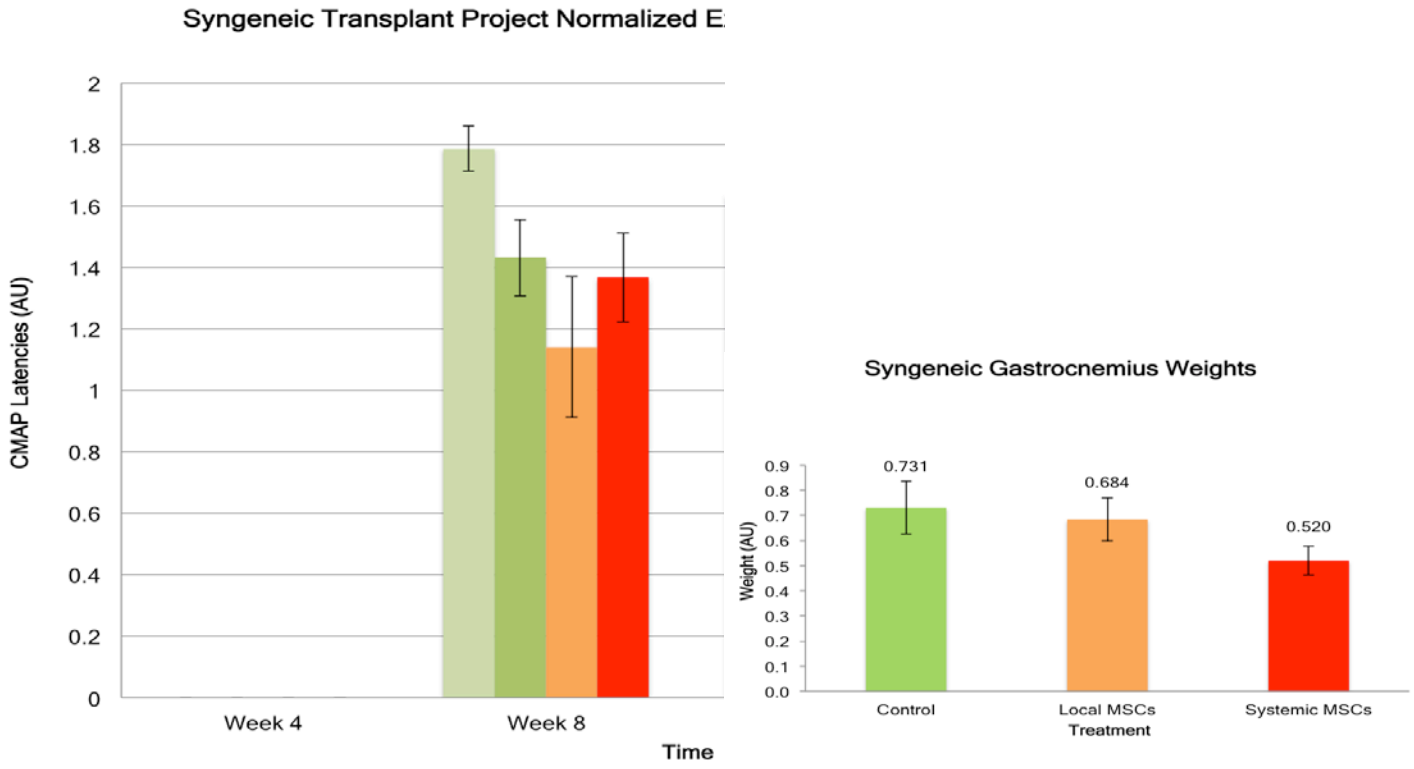


Figure 16 (left)**:** Syngeneic Hind Limb Transplant: Normalized Experimental CMAP Latencies Over time.
Figure 17 (right)**:** Syngeneic Hind Limb Transplant: Normalized Gastrocnemius Muscle Weights at 16 weeks

NORMALIZED GASTROCNEMIUS WEIGHT ANALYSES:
Gastrocnemius weights were measured for non-operated contralateral limbs and for the experimental limbs in all groups. Results are shown in Figure 17 with normalization of experimental limb gastrocnemius weight to contralateral non-operated limb. However, due to differences in weights between donor and recipient as well as altered growth curve following transplantation, no significant differences were noted using T-test statistical analysis.

NERVE HISTOMORPHOMETRY:
Histomorphometric analysis of experimental groups is depicted in Figure 18. The control group showed a higher number of small regenerating axonal sprouts with myelinated axons and lower overall density. However, the differences were not statistically significant when comparing experimental groups to control using difference of means testing.

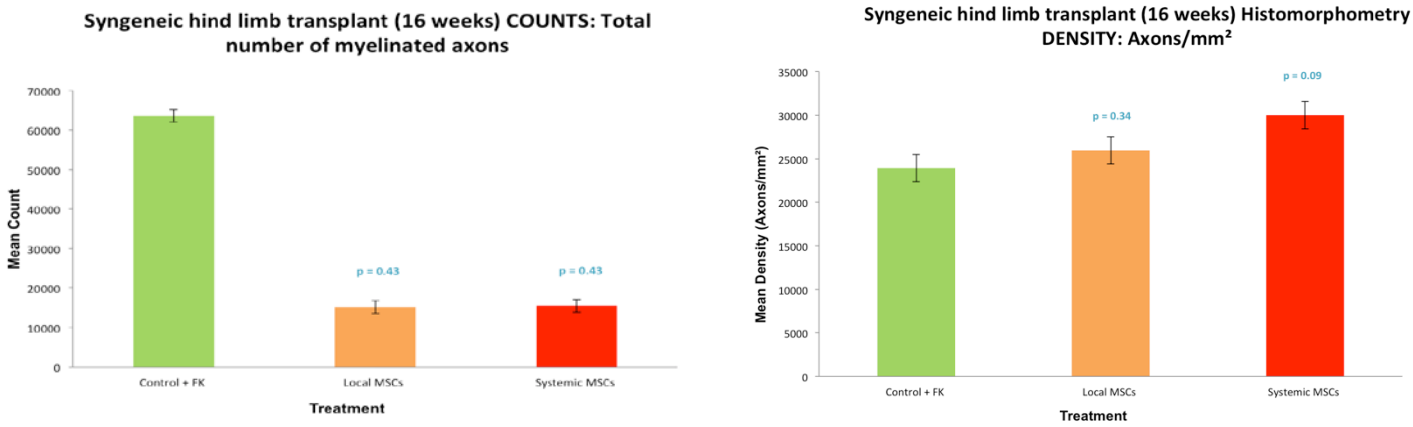


Figure 18: Syngeneic Hind Limb Transplant (16 weeks): A comparison Total Count of Myelinated Axons and Axon Density among Control, Local MSC and Systemic MSC treatment groups.

OVERALL SUMMARY:
Syngeneic hind limb transplants were performed with experimental groups that received local or systemic MSC injections and short-course subtherapeutic immunosuppression. Catwalk gait analysis indicated a small improvement in experimental paw positioning and intensity relative to control in the local MSC injection group at 16 weeks. Alternatively, the systemic MSC injection group was noted to have a significant increase in average maximum CMAP relative to controls at 16 weeks. Finally, similar to findings in Task 1, there were no significant differences noted among groups in histomorphometry or normalized gastrocnemius weight. High variability between animals and low numbers of total samples may have mitigated increased changes noted among groups. Despite these limitations, there was a clear objective trend toward improved function in groups that received MSC injections.

TASK 3: DETERMINE NEUROGENERATIVE AND IMMUNOMODULATORY EFFECTS OF MSC THERAPY IN ALLOGENEIC HIND LIMB TRANSPLANT

This set of experiments orthotopic hind limb transplants were performed from fully MHC mismatched Brown Norway to Lewis rats. Animals undergoing allogeneic hind limb transplants were divided into four groups: no treatment control group; short-term immunosuppression control group (0.5mg/Kg daily tacrolimus for 30 days); a local MSC experimental group ($50x10^4$ cells injected into distal stump

with tacrolimus for 30 days); and a systemic MSC experimental group (single dose of 10^6 million cells IV intra-operatively with tacrolimus for 30 days).

Functional and histological analysis was performed as noted above. In addition, graft survival analysis was performed using Kaplan Meier estimates.

COMPUTER-ASSISTED GAIT ANALYSIS USING CATWALK AND ELECTROPHYSIOLOGY:

A majority of animals in the allogeneic groups had advanced rejection prior to six weeks. From the syngeneic transplantation group experiments (Task 2), it is clear that significant functional recovery does not occur prior to 6-8 weeks. Therefore, functional outcome parameters were not measurable for most animals.

In addition, Normalized Experimental CMAP amplitudes and latencies obtained at early time points immediately prior to rejection (4 weeks) showed minimal return of function and variability limited statistical analysis (Figure 19). This is consistent with the syngeneic transplant project data (Task 2), where amplitudes were not measurable until 8 weeks.

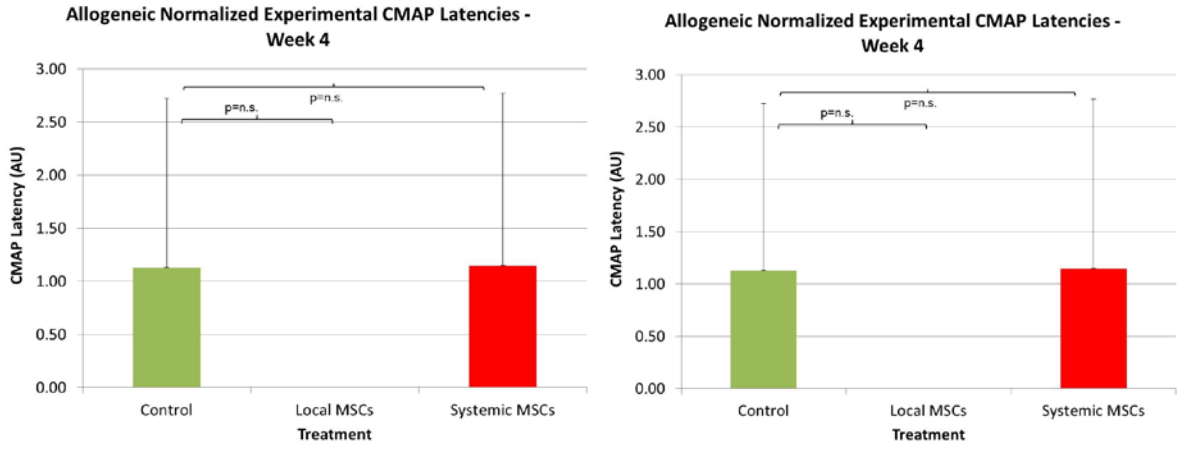


Figure 19: Allogeneic Hind Limb Transplant: Normalized Experimental CMAP prior to rejection (4 weeks post-transplant).

NORMALIZED GASTROCNEMIUS WEIGHT ANALYSES:

Gastrocnemius weights were measured for non-operated contralateral limbs and for the experimental limbs in all groups. Results are shown in Figure 20 with normalization of experimental limb gastrocnemius weight to contralateral non-operated limb. Data were not significant using difference of means testing. This is likely to due to variable degrees of edema from alloimmune response, making controlled comparison difficult and results inconclusive.

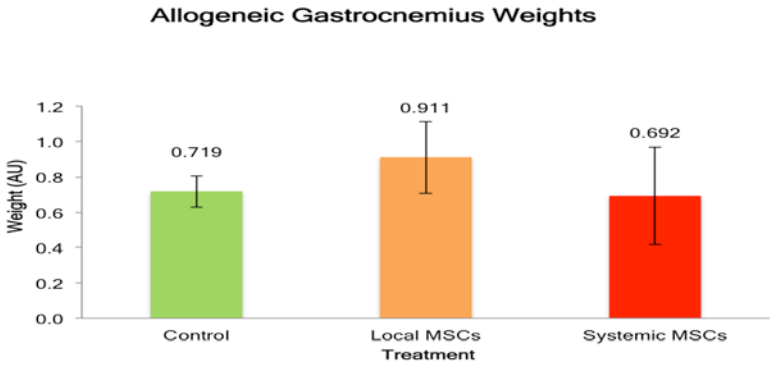


Figure 20: Allogeneic Hind Limb Transplant: Normalized Gastrocnemius Muscle Weights at 16 weeks

NERVE HISTOMORPHOMETRY:

Histomorphometric analysis of experimental groups is depicted in Figure 21. Overall, a small number of samples and significant variability resulted in a comparison of means between experimental groups and the control group that was not significant. However, there was a trend toward increased numbers and density of axons in the systemic MSC experimental group, but there was only borderline significance.

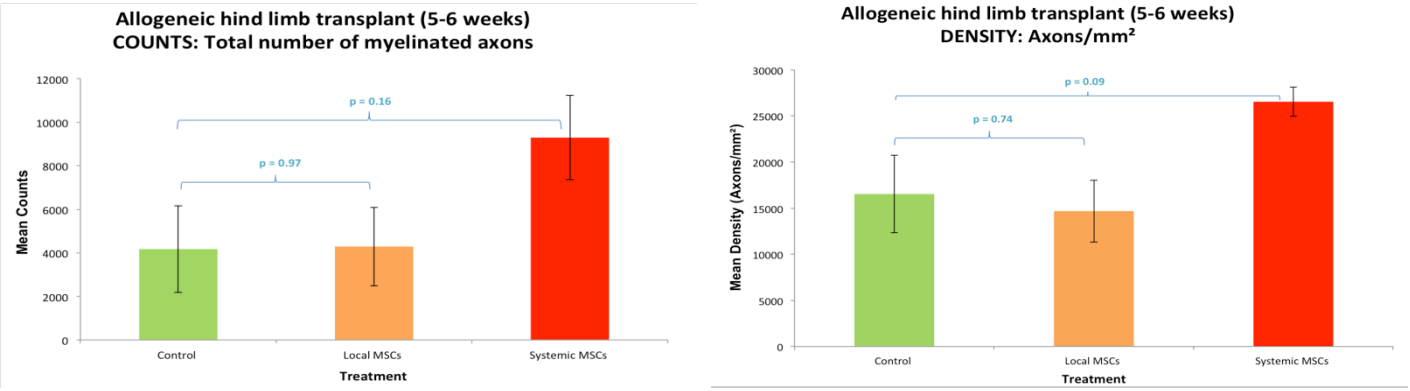
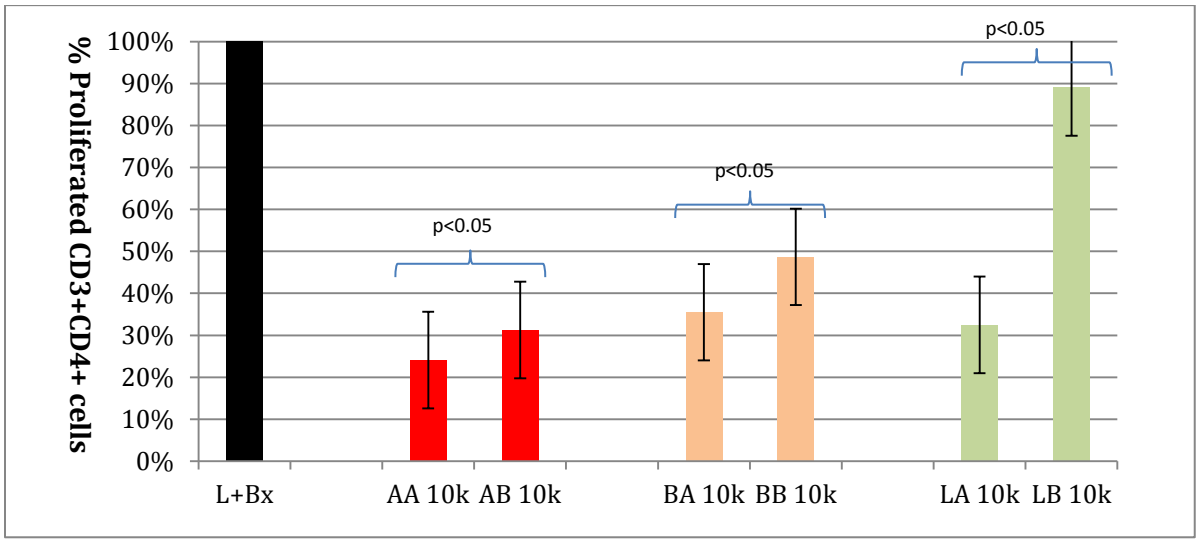


Figure 21: Allogeneic Hind Limb Transplant (16 weeks): A comparison Total Count of Myelinated Axons and Axon Density among Control, Local MSC and Systemic MSC treatment groups.

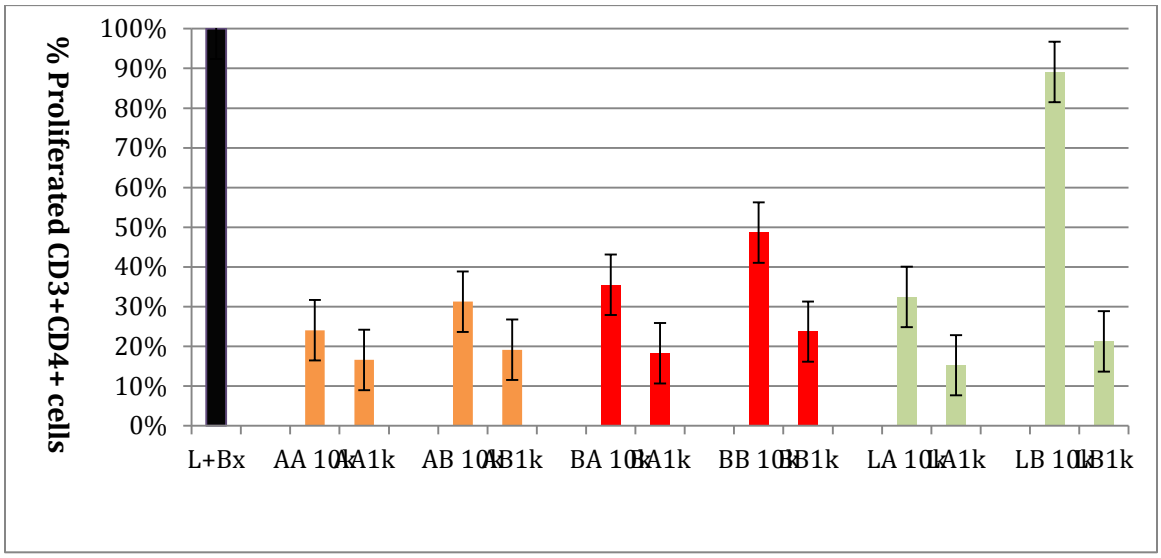
***IN VITRO* IMMUNOGENICITY AND IMMUNOMODULATORY PROPERTIES OF MSCS:**

In a mixed lymphocyte reaction (MLR), CFSE (carboxyfluorescein diacetate succinimidyl ester)-labeled Lewis rat splenocytes were cultured either 1) alone, 2) with irradiated Lewis splenocytes, 3) with irradiated Brown Norway (BN) rat splenocytes, with a number of 100,000 to 50,000 in a 96-well round bottom plate. or 4) with phytohaemagglutinin (PHA). Group 3 and 4 were further co-cultured with either varying doses of sorted BMSCs, or ASCs from 1:1,000 to 1:10,000 to assess for immunomodulatory effects. BMSCs and ASCs were isolated from Lewis, Brown Norway and ACI rats. Cell proliferation was examined on day 5 by Flow Cytometry analysis.

a.



b.



c.

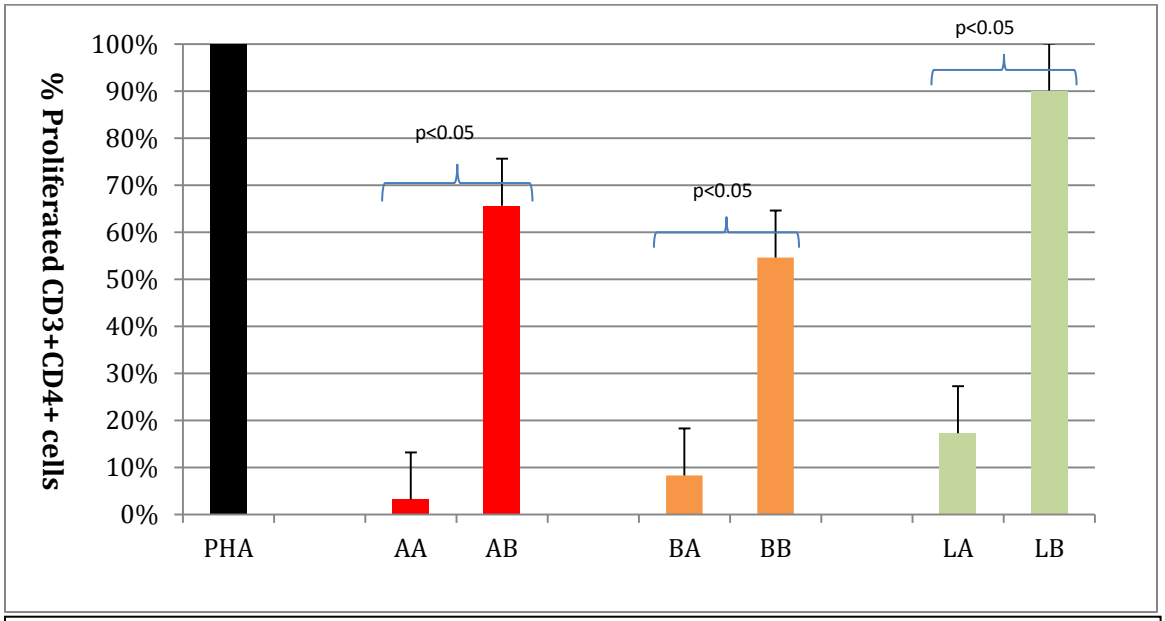


Figure 22:(a)Immunomudulatory effects of ASCs and BMSCs; (b) the dose-dependent manner of all MSCs; (c). MSCs regulating PHA-induced proliferation. * L: Lewis Splenocytes Bx: Irradiated BN Splenocytes. AA: ACI-ASCsAB: ACI-BMSCs; BA: BN-ASCs, BB:BN-BMSCs; LA: Lewis-ASCs, LB: Lewis-BMSCs

Both adipose-derived MSCs (ASCs) and bone marrow derived MSCs (BMSCs) displayed very low immunogenicity in MLR. When compared with stimulated cells alone, co-culture of responding splenocytes with both type of MSCs suppressed allogeneic stimulation (Figure 22a). Moreover, ASCs are superior than BMSCs ($p<0.05$) in all three stains (ACI, BN and Lewis) and the regulation showed dose-dependent manner of both ASCs and BMSCs (Figure 22b). Even at a ratio of 1:1000, MSCs can significantly suppress the non-specific proliferation of PHA (Figure 22c). Interestingly, allogeneic ASCs and BMSCs (either ACI or BN) showed better ability than autologous origin. ($p<0.05$)

IN VIVO IMMUNOMODULATORY PROPERTIES OF MSCS:

In addition to functional assays, we also tested the ability of MSCs to promote allograft survival and immunosuppression. A Kaplan-Meier survival curve for experimental and control groups is depicted in Figure 23. Due to highly immunogenic skin component of CTA, no immunosuppression (the no treatment control group) rejected allografts at day 7-9. Remaining groups rejected grafts on days 31-39. Despite potent immunomodulatory properties *in vitro*, experimental groups rejected allografts after cessation of tacrolimus maintenance therapy at rates similar to the no treatment group.

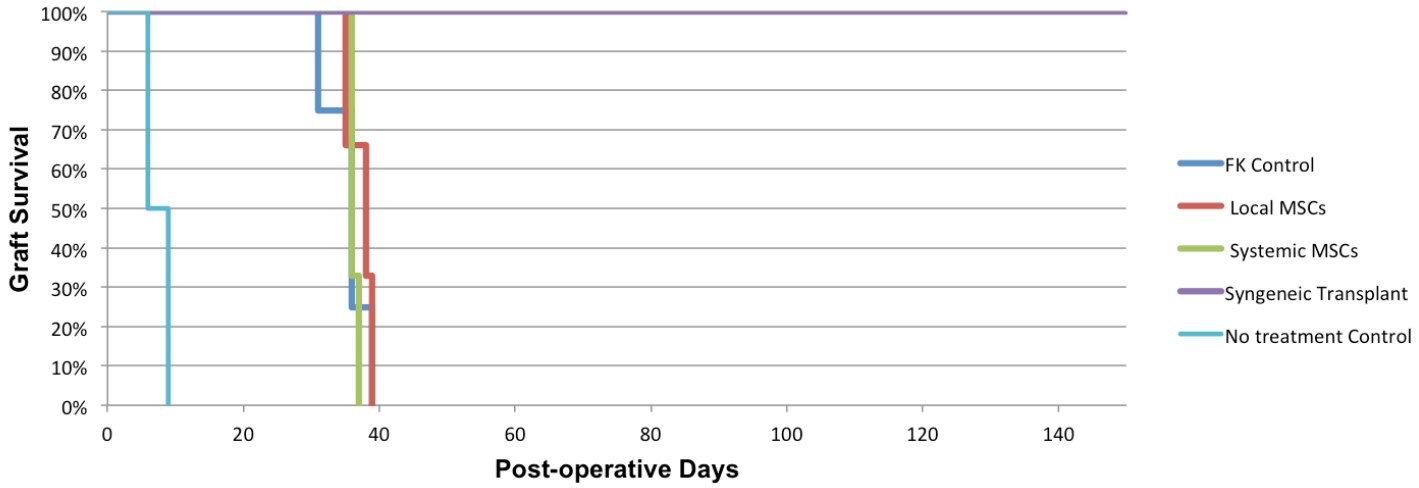


Figure 23: Kaplan-Meier Survival Curve: Rejection-free survival of skin component of CTA following allogeneic hind limb transplant and syngeneic hind limb transplant.

OVERALL SUMMARY:

In vitro mixed lymphocyte reaction showed potent immunoregulatory properties of adipose derived as well as bone marrow derived MSCs with the adipose derived cells demonstrating the most powerful immunoregulatory effects. Allogeneic transplants were then performed to determine the ability of local and systemic MSC injection to effect rejection. Animals were subjected to a short course of immunosuppression for 30 days. After cessation of immunosuppression, both experimental groups rejected within 2 weeks similar to control groups. Despite their ability to regulate immune reactions *in vitro* the cells did not delay or prevent rejection. Additionally, because of the early rejection of the grafts, there was no significant functional recovery noted on electromyography or Catwalk gait analysis. Similarly, no significant differences were noted on histology or gastrocnemius weight at this early time point. The discrepancy between *in vivo* and *in vitro* results as well as the limited functional recovery data warrants further study.

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KEY RESEARCH ACCOMPLISHMENTS

- Established MSC isolation and culture protocol.
- Demonstrated minimal immunogenicity and potent immunomodulatory properties of MSCs *in vitro* mixed lymphocyte reaction.
- Demonstrated superior immunomodulatory properties of adipose derived MSCs when compaired to bone marrow derived MSCs.
- Optimized surgical techniques and animal models for sciatic nerve repair, rat hind limb allotransplantation and cell delivery methods.
- Demonstrated the efficacy of MSC therapy for enhancing nerve regeneration using both inter-venous and direct nerve injection.
- Demonstrated the neuroregenerative effects of MSC in limb transplantation in the absence of alloimmune response.
- Demonstrated the neuroregenerative and immunomodulatory properties of MSC in the presence of alloimmune response.

REPORTABLE OUTCOMES

INTERNATIONAL PRESENTATIONS:

Wu L, Yuan N, Rubin JP, Christensen J, Ibrahim Z, Lee WP, Schneeberger S, Cooney DS, Brandacher G. Comparing the Immunoregulatory Effects of Bone Marrow- and Adipose-Derived Mesenchymal Stem Cells. Poster Presentation, 24th International Congress of the Transplantation Society Poster Sessions, Jul 15th-19th, 2012 Berlin, Germany.

Wu L, Yuan N, Rubin JP, Grahammer J , Christensen JM, Ibrahim Z, Lee WP, Sacks JM, Brandacher G, Cooney DS. Comparing the Immunoregulatory Effects of Bone Marrow- and Adipose-Derived Mesenchymal Stem Cells, 10th Annual Meeting of the International Federation for Adipose Therapeutics and Science, Oct. 5-7th, 2012 Quebec City, Quebec, Canada (accepted for Podium Presentation)

REGIONAL PRESENTATIONS:

Wimmers EG, Brandacher G, Lee WP. Stem Cell Therapy for Nerve Regeneration and Immunomodulation After Composite Tissue Allotransplantation. Poster presentation, Ohio Valley Society, Robert Ivy Society and Maryland Society of Plastic Surgeons Conference, White Sulphur Springs, WV, June 2011.

W.P. Andrew Lee, Cooney D, Wimmers EG, Ibrahim Z, Brat G, Brandacher G. Mesenchymal Stem Cell Therapy for Nerve Regeneration and Immunomodulation after Composite Tissue Allotransplantation. Plenary Presentation, Military Health System Research Symposium (MHSRS), Fort Lauderdale, FL, August 13, 2012

CONCLUSION

Mesenchymal Stem Cells enhance functional recovery following nerve injuries and limb transplantation. Mesenchymal stem cells also possess potent immunomodulatory properties with minimal immunogenicity *in vitro*. However, due to highly immunogenic skin component of CTA in a stringent fully mismatched strain combination, the immunomodulatory properties could not be demonstrated *in vivo*. Further studies are warranted to explore mechanistic relationships of MSC therapy in nerve regeneration and to optimize dosage and frequency of MSC therapy for targeted immunomodulation in CTA.

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APPENDICES

Published Abstracts:

Comparing the Immunoregulatory Effects of Bone Marrow- and Adipose-Derived Mesenchymal Stem Cells

Wu L.[1], Yuan N.[1], Rubin P.[2], Lee A.[1], Brandacher G., Cooney D.S.[1],

[1]Johns Hopkins Medical Institutions, Plastic Surgery, Baltimore, United States, [2]University of Pittsburgh Medical Center, Surgery, Pittsburgh, United States

Background: Both bone-marrow (BMSCs) and adipose-derived (ASCs) mesenchymal stem cells have recently been described to be a promising strategy to promote transplant tolerance. However, the unique immunoregulatory properties of BMSCs and ASCs and their individual ability to suppress an allo-immune response have not been directly compared yet.

Methods: BMSCs and ASCs were isolated from Lewis, Brown Norway (BN), and ACI rats. Cells were expanded and sorted. In a mixed lymphocyte reaction (MLR), CFSE-labeled Lewis rat splenocytes were cultured either 1) alone, 2) with irradiated Lewis splenocytes, 3) with irradiated BN rat splenocytes, or 4) with phytohaemagglutinin (PHA). Group 3 and 4 were further co-cultured with either varying doses of sorted BMSCs, or ASCs or media harvested from stem cell cultures to assess for immunomodulatory effects; also for Group 3, besides routine regulation, the MSCs were added 3 days after the initial alloreaction (Delayed regulation), or taken away after 3-day of co-culture (Interrupted regulation). Cell proliferation was examined on day 5 by FACS analysis.

Results: BMSCs and ASCs of all strains showed similar functional and immnunophenotypical properties, and displayed low immunogenicity comparable to non-stimulated controls. All allogeneic co-culture MLRs showed vigorous cell proliferation as compared to cultured cells from Groups 1 and 2.When added to allogeneic MLRs, both BMSCs and ASCs of different origins (recipient, donor and third party) significantly suppressed allogeneic and PHA stimulation in a dose-dependent manner. Media alone extracted from stem cell cultures, however, did not suppress proliferation of mixed lymphocytes in any group indicating likely contact-dependent regulatory mechanisms of MSCs. Moreover, in both delayed and interrupted regulation, the suppressive effects diminished to a very limited level.

Conclusion: We have performed for the first time a head-to-head comparison of the immunoregulatory potential of BMSCs and ASCs using in vitro cell culture. MSCs from different tissue origins exert immunomodulation in allo- and in PHA-induced stimulation, indicating immunoregulatory effects of this type of stem cells are non-specific and the process requires a sustaining contact. With the evidence collected so far, ASCs seem to have superiority in suppressing non-specific regulation than BMSCs.

MESENCHYMAL STEM CELL THERAPY FOR NERVE REGENERATION AND IMMUNOMODULATION AFTER COMPOSITE TISSUE ALLOTRANSPLANTATION

W.P. A. LEE, MD, G. BRANDACHER, MD, D. COONEY, MD, PHD,
E. WIMMERS, MD, G. BRAT, MD, Z. IBRAHIM, MD

PURPOSE/AIMS: Composite Tissue Allotransplantation (CTA) such as hand and face transplantation is a clinical reality. However, widespread clinical application of CTA for catastrophic combat trauma will only be realized if immunosuppressive risk is reduced and functional outcome is maximized. The implementation of cellular therapies that integrate the concepts of transplantation tolerance with those of tissue regeneration could fine tune current immunomodulatory approaches as well as optimize functional outcome of these reconstructive modalities. Our study proposes a novel cell-based therapy utilizing systemic administration of mesenchymal stem cells (MSC) that can augment nerve regeneration while minimizing the need for immunosuppression.
DESIGN: Quantitative basic science research was performed using animal models of limb transplant and nerve repair for efficacy and feasibility studies using MSC's to improve outcomes.
POPULATION/SAMPLE STUDIED: Rodent models of sciatic nerve injury and hind- limb transplantation.
METHODS: Translational studies in small animal models of sciatic nerve transection and repair (Lewis rats), syngeneic (Lewis to Lewis) and allogeneic (Brown Norway to Lewis) hind limb transplants were performed to test neuroregenerative and immunomodulatory properties of MSCs. Functional Outcome analyses were performed using computer assisted gait based studies (Catwalk System, Noldus Technology), electrophysiology (ADInstruments) and nerve histomporphometry. Immunomodulatory properties were analyzed using mixed lymphocyte reaction.
DATA ANALYSIS: Student's t test was utilized for continuous variables
FINDINGS: In the Sciatic nerve injury MSC administration demonstrated improvements in nerve recovery measured by maximum compound muscle action potential (CMAP). Mean values for maximum CMAP amplitudes of control, local and systemic MSC injection with standard deviation were 0.45 +/- 0.151, 0.44+/-0.10, and 0.67+/-0.24, respectively ($p<0.1$). Gastrocnemius weight ratios (experimental/contralateral un-operated) for control, local and systemic injection were 0.72, 0.63, and 0.84, respectively. Nerve fiber density was measured using Histomorphometric data and comprehensive gait-based analysis was performed using the Catwalk system.
CONCLUSIONS/RECOMMENDATIONS: Systemic delivery of MSC improves nerve regeneration and will improve reconstructive transplant outcomes. Current studies will determine the immunomodulatory effects of this treatment as well.
IMPLICATIONS: Innovative cell based approaches can potentially favor the risk benefit ratio of reconstructive transplantation towards better functional recovery of our wounded warriors.
FROM/TO TIME PERIOD OF STUDY: 2011-2012
FUNDING: CDMRP Hypothesis Award

Stem Cell Therapy for Nerve Regeneration and Immunomodulation After Composite Tissue Allotransplantation.

Wimmers EG, Brandacher G, Lee WP.

Composite tissue allotransplantation (CTA) is hampered by inadequate nerve regeneration and difficulties balancing immunosuppression. We utilize a novel cell-based therapy comparing bone marrow derived mesenchymal stem cells (BM-MSC) and adipose derived stem cells (ASC), to improve nerve regeneration and also enhance immunomodulation. To date, neither BM-MSC nor ASC have been tested in a CTA model to demonstrate both effects. The stem cells are harvested and cultured from Brown Norway rats, and subsequently tested for CD29 and CD90 to measure total purity. Administration of stem cells occurs via either direct injection into the distal stump of the transected nerve, or comparatively by systemic injection (intravenous). Preliminary results show increased nerve growth in the stem cell groups compared to controls, as determined by video gait kinematics, nerve conduction velocity, and gastrocnemius weight. Further testing is in progress. These early data indicate that BM-MSC and ASC may serve as a useful adjunct to accelerate nerve regeneration